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SOME PROBLEMS IN QUANTITATIVE ANALYSIS WITH CONCENTRATION-SENSITIVE DETECTORS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Basic problems that arise in quantitative analyses in high-performance liquid chromatography with concentration-sensitive detectors are discussed. The linear range of a spectroscopic (UV) detector is, among other parameters, a function: (1) of the wavelength and its distance from the maximum of the absorbance, and (2) the bandwidth of the monochromator or filter. Experimental methods are proposed for determining the short- and long-time averaged flow-rates and the reproducibility of the sampling system. The short-time averaged flow-rates were measured with four commercially available types of equipment, and the variations of the results were between ± 0.6 and $\pm 1.0\%$. In routine quantitative analysis a reproducibility of $\pm 1\%$ is a desirable aim. The use of internal standards does not decrease these limits. The results with gradient elution method were worse.

INTRODUCTION

The problems that arise in quantitative analysis in high-performance liquid chromatography (HPLC) using peak areas have rarely been discussed in detail[1–3], although they are similar to those which are well known in gas chromatography. The difficulties in liquid chromatography (LC) are greater because (a) concentration-sensitive detectors are used almost exclusively and (b) the concentration of the sample in the eluent at the end of the column is smaller by roughly a factor of 100 or more than in gas chromatography with packed columns. Consequently, very sensitive detectors are required in HPLC.

Concentration-sensitive detectors

Concentration sensitive detectors[4], like UV and refractive index detectors, are those in which, at a constant pressure and temperature, (1) the detector signal, S, depends on the concentration of the sample, c, in the eluent, (2) the detector signal is independent of the mass flow-rate of the sample (for example, if the flow of the eluent is stopped, the signal of the detector remains more or less unaffected), and (3) the sample undergoes no chemical change by the measurement, *i.e.*, it can be recovered from the detector effluent.

It should be mentioned that the moving-wire (or chain) detector[5] is also a concentration-sensitive detector (*i.e.*, the dimension of the peak area in a chromatogram is time and not the mass of the sample, as discussed later), because the mass transfer of the eluent (including the dissolved sample) from the outlet of the column to the detector is constant, although the detector itself (*i.e.*, flame-ionization detector) can be a mass flow-rate sensitive detector. However, conditions (2) and (3) above are not fulfilled in this instance.

The mass flow-rates of the sample, F_1, and that of the eluent, F, are given in units of grams (or moles) per unit time. The concentration of the sample in the eluent:

$$c = \frac{F_1}{F_1 + F} \tag{1}$$

is expressed in non-dimensional units.

The concentration of a sample in the eluent at the peak maximum can be calculated for an efficient column packed, for example, with 10-μm silica, where $h = 50$ μm. The column is 30 cm in length with I.D. 4 mm, its total porosity is 0.8 and the density of the eluent is 0.8 g/ml. The sample size is 1 μg per compound and the samples have capacity ratios (k') of 0 (inert) and 2. It is assumed that 4 ω volume units of the eluent include all of the sample (where ω is the standard deviation of the peak in volume units of the eluent) and that the concentration at the peak maximum is about the twice the average concentration of the sample in the eluent at the end of the column and in the detector itself. The calculated (and measured) concentrations are $16 \cdot 10^{-6}$ g/g and 5.3 ppm for the inert and for a retarded peak ($k' = 2$), respectively. In routine HPLC these concentrations are almost always less than 100 ppm. Consequently, in eqn. 1 $F_1 \ll F$ and

$$c = \frac{F_1}{F} \tag{2}$$

can be used with advantage.

Linearity of the detector

A concentration-sensitive detector has a linear response if the signal, S, is proportional to the concentration of the sample in the eluent:

$$S = r \cdot c + \text{constant} \tag{3}$$

where r is the response factor (or response). This range has to be determined by measuring the signal of the detector as a function of known concentrations of the sample in the eluent[6].

Solutions with well defined concentrations of the sample in the eluent have to be prepared and the cell of the detector has to be flushed with these solutions. The signal of the detector is then determined as a function of the concentration under static and dynamic conditions.

The linear range of a detector is a function of its geometry, of the quality of the amplifier, etc. At high concentrations, the signal of an optical detector is not proportional to the concentration, because the solution is not infinitely diluted, *i.e.*,

the Lambert–Beer law is not obeyed. The linearity range is also defined by the maximum, arbitrarily allowed, deviation from the ideal value.

According to another approach, the determination of the "linearity" of a detector is based on the relationship between the sample size and the peak area[7]. The results obtained with this method reflect the characteristics not only of the detector itself, but also of the entire chromatographic equipment. With samples of identical size, the concentration of the sample in the eluent at the peak maximum is higher for peaks with shorter retention times (*i.e.*, smaller capacity ratios) than for those with higher k' values. It is conceivable that the concentration pertinent to a high peak may be outside the linearity range, while with a flat peak the linearity range is not exceeded. On the other hand, it must be borne in mind that calibration with the conventional parameters of HPLC (*i.e.*, sample size and peak area) is a definite advantage for the analyst.

If the linearity of a detector is determined by the first method (*i.e.*, with eqn. 3), sometimes the deviations from the ideal value as a function of the concentration of the sample in the eluent are either positive or negative. This "alternating effect" was described over 10 years ago for a flame-ionization detector in gas chromatography[6]. The consequence of the alternating effect may be that the deviation from linearity is positive, negative or zero, depending on the concentration at the peak maximum, although the concentrations are definitely outside the linear range, if the linearity of the detector is determined by the peak area method.

If optical detectors are used it should be borne in mind that the Lambert–Beer law is valid only for infinitely dilute solutions:

$$\log (I/I_0) = -\varepsilon cd = -a \tag{4}$$

where I_0 is the light intensity when the cell of the detector is filled with the eluent, I is the light intensity when the concentration of the sample in the eluent is c, ε is the extinction coefficient and d is the thickness of the cell. In our experience, if a UV detector ($d = 0.5$ cm) at a wavelength of 254 (± 8) nm is used to determine the concentration of benzene in a practically non-absorbing eluent, the end of the linear range is at an absorbance, a, of *ca.* 1, which corresponds to a concentration of about 0.1% (v/v) of benzene in the eluent at room temperature[8]. From our experience with other samples, the upper concentration limit was between 100 and 1000 ppm, more or less independent of the nature of the sample. The extinction coefficient of benzene at the given wavelength ($\varepsilon = 200 \cdot 10^3$ ml·mol^{-1}·cm^{-1}) is less than the average value. The maximum allowed absorbance for samples with lower ε values or with a smaller thickness of the cell is, of course, smaller. It is often forgotten that the upper end of the linearity of an optical detector is usually not determined by the linearity of the optics and that of the amplifier, but mostly by the high concentration of the sample in the eluent.

The monochromators or filters in UV detectors in HPLC have a more or less broad bandwidth. The signal-to-noise ratio increases with increasing bandwidth of the filters, because noise and drift are caused mainly by the receivers, *i.e.*, photodiodes. The linear range of the detector, however, decreases if inside this bandwidth the extinction coefficient, ε, is not constant[9]. The absorbances, with different ε values as a function of the wavelength, inside the bandwidth have to be summed. From eqn. 4,

the logarithm of the light intensity, I increases with decreasing absorbance, a. The consequence of this summation is that the appropriate transformed signal of an optical detector will not be a linear function of the concentration, c, if ε is not constant inside the bandwith[9].

For example, if quantitative analysis of condensed aromatics with a UV detector is to be carried out, the maximum of the absorption shifts from 254 nm for benzene to 308 nm for coronene. The wavelength of the UV detector has to be shifted for every component so as always to be at the maximum of the absorption band, if exact quantitative analysis is required. Otherwise, a further correction factor that is a function of the concentration of a particular sample in the eluent has to be used, because of the deviation from linearity of the detector response. This possible error can be checked by determining the range of the linearity for every compound at the fixed wavelength of the detector. The error increases, of course, with increasing $\Delta\varepsilon$ for the different samples at a constant wavelength.

A further problem in quantitative work may be that the sensitivity of the receiver itself is a function of the wavelength[9].

The noise level and drift of commercially available UV detectors correspond optimally to an absorbance of $a = 10^{-4}$ or slightly better. The noise of multi-wavelength UV detectors is usually greater by a factor of 10, and consequently the linearity range is 1:1000 and sometimes 1:5000 in routine analysis. Double-beam instruments have some advantages, but they do not give any improvement in noise level and drift over single-beam instruments because both are mainly caused by the photodiodes[8].

Sample–peak area relationship

Quantitative evaluation of chromatograms requires the determination of the peak areas, A, *i.e.*, integration of the detector signal with respect to time. The concentration of the sample in the eluent, c, can always be expressed in non-dimensional units, and consequently A in this instance has the dimension of time. Combining eqns. 2 and 3, we obtain

$$A = \int_0^t S_i \mathrm{d}t = r \int_0^t c_i \mathrm{d}t = \frac{r}{F} \int_0^t F_i \mathrm{d}t = \frac{rm_i}{F} \tag{5}$$

because the integral of the mass flow-rate of the ith sample, F_i, with respect to time is the mass, m_i, of the first sample component injected. Rearranging eqn. 5:

$$m_i = \frac{F}{r_i} \cdot A_i \tag{6}$$

A quantitative evaluation of a chromatogram pre-supposes proportionality between the peak area, A, and the mass of the corresponding sample component, m_i. As can be seen from eqns. 5 and 6, this is true only for a constant flow-rate of the eluent, F. Further, it follows from eqn. 5 that the peak area, A, is inversely proportional to the flow-rate of the eluent, F. Any deviation from a given constant flow-rate of the eluent during the period the separated peaks are in the detector, and only during this period, will result in erroneous results in the quantitative analysis. In other words, fluctuations in F during the periods when peaks do not emerge from the detector do

not give erroneous quantitative results. Consequently, in quantitative work only the short-time averaged flow-rates (*i.e.*, during the elution of the peaks) and their accuracy and reproducibility are of interest.

In routine HPLC, the flow-rate is usually determined by measuring the volume (or mass) of the emerging eluent at the outlet of the detector. From experience, F can be measured with an error of $\pm 1\%$ if relatively large aliquots are collected. Consequently, the flow-rate is averaged over a relatively long period of time while the peaks and baseline can be observed on the chromatogram. The averaging of F can simulate a better or worse constancy than that during the elution of the peaks, and only this is of interest. This effect could be considerable with flow-rate-controlled pumps because of the time delay between the measured signal (*i.e.*, F or pressure) and the mechanical adjustment of the pump itself.

In quantitative HPLC, the dead volume of the detector cell has to be small compared with the eluent volume, 4ω, in which the sample is dissolved. If a column is packed with 10-μm silica, an h value of 50 μm seems to be typical. If the inner diameter of a column of 20 cm length is 4 mm, the volume of the eluent in which the inert sample is dissolved will be *ca.* 130 μl and it increases with $(1+k')$ for a retarded peak. In up-to-date analytical equipment, the cell volume is *ca.* 10 μl, *i.e.*, it is small enough. It should be borne in mind that 4ω is proportional with $\sqrt{hL}$ (where L is the length of the column), but it decreases with the square of the decreasing inner diameter of the column. The dead volume of the cell gives rise to an error only if the I.D. of the column becomes much less than 4 mm. On the other hand, from experience, the efficiency of a column (packed with well known methods) decreases sharply for I.D. < 3 mm. A decreasing particle size, d_p, of the support is not as important, because if $d_p < 10$ μm and the linear velocity is not extremely high, then h is proportional to the particle size, to a first approximation.

Another source of error in quantitative work is the noise level and drift of the system, which may be caused by the instability of the detector itself or by impurities in the eluent. The error is a function of the signal-to-(noise plus drift) ratio and is of especial interest if the peaks are tailing.

The error due to the limited resolving power of the electronic integrators is usually negligible in HPLC compared with the other methods if up-to-date commercially available units are used, including relatively simple units.

To summarize, the error or reproducibility in quantitative analysis is always greater than (or optimally identical with) the short-time stability of the flow-rate. Sometimes the errors may compensate each the other. In this paper, a simple method is described for measuring the short-time constancy (*i.e.*, during the elution of a single peak) of the flow-rate.

Short-time averaged flow-rate

In the following discussion, all sources of errors in quantitative analysis, except that of the flow-rate, will be avoided. Only clean eluents were used. The samples were well resolved on the non-"bleeding" stationary phase at constant temperature ($30 \pm 0.1°$) and their concentrations in the eluent at the peak maxima were similar. The signal-to-noise ratio was high. The standard deviation of the reproducibility of the peak areas for a given compound was produced mainly by the short-time fluctuation of the flow-rate and the reproducibility of the sample size.

It will be assumed that the peaks are triangular in shape. Essentially the same deliberations apply to peaks with other constant shapes, *e.g.*, gaussian curves. The height of this isosceles triangle is b (concentration units) and its width is w, where w is four times the standard deviation of the original gaussian peak in time units. The sample is now dissolved in a volume V_s, where

$$V_s = wF \tag{7}$$

and

$$A = \frac{1}{2} bw = \frac{bV_s}{2F} \tag{8}$$

Then, if V_s = constant, the relative error of the peak area is

$$\frac{\Delta A}{A} = \frac{\Delta F}{F} + \frac{\Delta b}{b} \tag{9}$$

If all the other parameters are kept constant and the detector response is linear, the peak height is proportional to the mass, m, or volume, V_s, of the sample injected and

$$\frac{\Delta A}{A} = \frac{\Delta F}{F} + \frac{\Delta m}{m} \tag{10}$$

It can be seen from eqn. 10 that the deviations in the peak areas are a consequence of both the variation of the flow-rate and the variation of the size of the injected sample.

Let the sum of all of the peak areas in a chromatogram be ΣA; then the reduced area of the ith peak, α_i, is

$$\alpha_i = \frac{A_i}{\Sigma A_i} \tag{11}$$

where $\Sigma \alpha_i$ is usually normalised to 100%. The reduced peak area, α, is independent of the sample size if the detector response is linear and the variations of the size of the injected sample are small. Consequently, from eqns. 10 and 11:

$$\frac{\Delta \alpha}{\alpha} = \frac{\Delta F}{F} \tag{12}$$

A given mixture is separated several times with unchanged conditions and the reduced areas, *i.e.*, the α_i values, for a given component are determined. As can be seen from eqn. 12, the relative error in α_i is identical with the relative variation of the flow-rate during the period when the ith peak is in the detector. For example, the standard deviation of the α_i values divided by the average value of α_i (*i.e.*, the relative standard deviation) is identical with the relative variations of the flow-rate, for example as a percentage. The variation of the flow-rate calculated in this way is characteristic for the time period during the elution of a particular peak. Of course, this procedure can be carried out with each component of a mixture. With increasing capacity ratios, k', the peaks become broader, and consequently the flow-rate of the

eluent is averaged over a longer period. As discussed above, tailing of the peak of a given compound can increase the standard deviation of α. From experience, the standard deviations of the flow-rate are more or less independent of the k' value of a given compound and of the average flow-rate of the pump if extreme conditions are avoided.

EXPERIMENTAL AND RESULTS

Chromatographic systems

Different types of equipment were used. The first group (I) included commercially available equipment in which constant flow-rates were achieved with electronic feedback. In the second group, the inlet pressure of the eluent was kept constant (IIa) or a metering pump was used (IIb). All pumps except one were membrane-type units. Home-made columns were used. Except with unit Ib, the same, simple electronic integrator (Model CRS 108, Infotronics, Techmation, Düsseldorf, G.F.R.) and a 10-mV recorder with a full-scale deflection time of 0.5 sec (Kompensograph III, Siemens, Karlsruhe, G.F.R.) were always used.

Ia. All units were obtained from Waters Assoc. (Milford, Mass., U.S.A.). The equipment included two pumps (Type 6000A), a programming unit (Type 660), a sampling system (U6K) and a UV detector (Type 440).

Ib. This was a Model 1084A liquid chromatograph (Hewlett-Packard, Böblingen, G.F.R.), including an integrator unit.

IIa. This was home-made equipment[10,11]. A membrane pump with three heads (Orlita, Giessen, G.F.R., Type M3-S4-4-4) produced pressures of up to 400 atm. The inlet pressure of the column was controlled by a self-built unit[10], which also de-gassed the eluent, smoothed the pulses, transduced from liquid to gas pressure and was the safety valve for the chromatographic system. The sample was injected on the top of the stationary phase[12] with a 10-μl syringe (Hamilton, Reno, Nev., U.S.A., Type 801-N). The peak broadening in the geometrically deformed connecting tubes[13,14] between the outlet of the column and the detector was minimal. The cell volume of the home-made UV detector was 4.5 μl, its dead volume including the tubing was 7 μl and the light path was 5 mm. The noise of the detector at 254 $\pm$ 8 nm corresponded to $5 \cdot 10^{-5}$ absorbance units if the temperature of the cell was stabilized.

IIb. This was home-made equipment, identical with IIa except for the use of a membrane-type pump with two heads (Orlita, "Mikrodosierpumpe", Type AE 10.4-4) and a pulse damping unit (Orlita, Type PD 4-500). The flow-rate of this pump, as in equipment IIa, was "constant", but it was not controlled by other means.

Sampling systems. The various equipment described above was used to determine the variation of the flow-rates. During the separations, the pneumatic resistance and the total porosity of the chromatographic column had to be kept constant, otherwise the retentions were not reproducible. Consequently, there was in principle no difference whether the flow-rate or the inlet pressure of the column was kept constant. From the point of view of routine work, however, it is recommended that constant flow-rate units are used.

If the inlet pressure is constant, septum or valve injection can be used. In routine work, the pneumatic resistance of the "column" changes because fine pieces of the septum are cut off by repeated injections. The smaller the particle size of the

support, the greater is the pneumatic resistance of the column and the effect described above becomes negligible. Unfortunately, sometimes thin films are produced with some eluents and septa and the pneumatic resistance even of columns packed with small particles increases sharply.

If the sampling system is any kind of a valve, there is always a by-pass and therefore the pneumatic resistance and the flow-rate changes as a result of the sampling.

If the sample size is large and its viscosity is high (*e.g.*, polymers), the change in the average viscosity of the eluent due to sampling is large and the flow-rate changes during the separation. This effect is independent of the nature of the sampling system.

Sample size. The sample mixture was dissolved in methanol and concentrations were chosen so as to achieve similar peak heights in the chromatogram. The concentration of the compounds in the mixture increased from *ca.* 50 to 100 ppm with increasing retention time. Using equipment Ia and Ib always 10 μl of sample solution and with IIa and IIb 5 μl of sample solution were injected.

Column. The column, 30 cm in length, was made of drilled[11] stainless-steel tubing (German Type No. 4571, equivalent to SS 316 in the U.S.A.) with I.D. 4.2 mm and O.D. 6 mm.

Eluent. The eluent was methanol–water (9:1, v/v). Methanol of "pro analysi" or "for pesticide analysis" grade (Merck, Darmstadt, G.F.R.) was used. The water was distilled twice. The viscosity of the eluent mixture was 1.02 cP at 30°. All measurements were made at 30 $\pm$ 0.1°.

Stationary phase and column packing. A reversed-phase (RP) stationary phase with octadecyl groups (RP-18) was used[15]. The silica support had an average pore diameter of 100 Å, a specific surface area of 350 m^2/g and a pore volume of 1 ml/g. The carbon content of this RP-18 was 18.1% (w/w), and the average spatial requirement of an organic bristle was about 60 $Å^2$. The balanced density packing method was used as described previously[11]. The packing density of these reversed phases is higher than that of "naked" silica and is *ca.* 0.5 g of RP per millilitre of empty column volume. The total porosity of the RP was 0.68 with a pore porosity of 0.26. The nominal particle size (d_p) of the silica, as stated by the producer, was 10 μm. The specific permeability of a column packed with the RP was $1.3 \cdot 10^{-9}$ cm^2, corresponding to a calculated particle size of about 11 μm (ref. 16).

Inlet pressure. The maximum inlet pressure of the column (30 cm $\times$ 4.2 mm I.D., d_p = 11 μm) was less than 200 atm at the maximum flow-rate used, *i.e.*, F = 6 ml/min or u = 12 mm/sec. Up to these pressures the sample was injected manually on to the top of the column with a syringe if the equipment IIa and IIb were used. With the sampling system described above at pressures up to 200 atm there were no problems in injecting the sample.

Efficiency. The efficiency of the separation column is demonstrated in Fig. 1. The h *versus* u curves are typical rather than excellent. It is usual with RPs and a polar eluent (mixture) that the efficiency decreases with increasing capacity ratios of the compounds. The data in Fig. 1 can be described in the given linear velocity range by the equation

$$h = A' + C'u \tag{13}$$

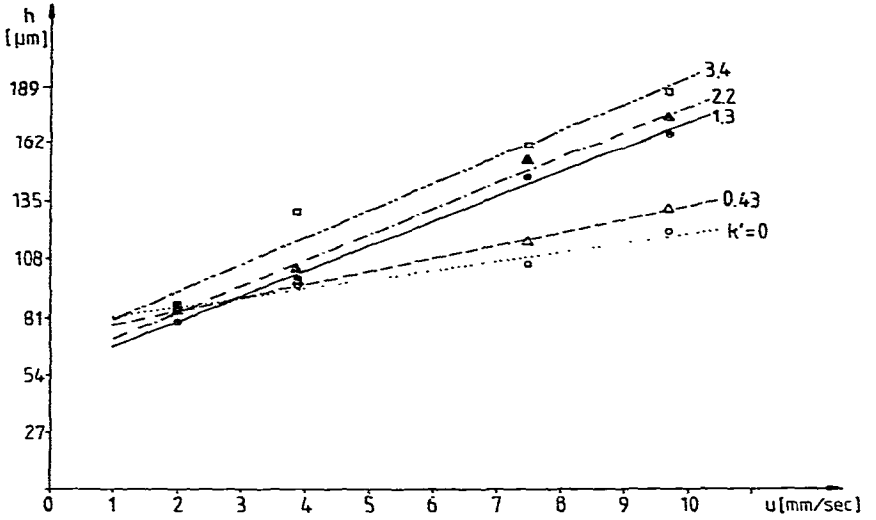

Fig. 1. *h versus u* curves on C_{18} RP stationary phase. $d_p = 11\ \mu m$; I.D. = 4.2 mm, drilled; length = 30 cm. Sample size: 0.5–1 μg per compound. Eluent: methanol–water (9:1, v/v). Samples: 1 (○), nitromethane ($k' = 0$; $A = 76\ \mu m$; $C = 4.3$ msec); 2 (△), naphthalene ($k' = 0.43$; $A = 71\ \mu m$; $C = 6.2$ msec); 3 (●), pyrene ($k' = 1.28$; $A = 55\ \mu m$; $C = 11.7$ msec); 4 (▲), chrysene ($k' = 2.21$; $A = 59\ \mu m$; $C = 12.1$ msec); 5 (□), 3,4-benzfluoranthene ($k' = 3.37$; $A = 67\ \mu m$; $C = 12.6$ msec). $K = 1.3 \cdot 10^{-9}$ cm². UV detector (254 ± 8) nm.

where h is calculated in microns if A' is given in microns, C' in milliseconds and u in millimetres per second. The constants A' and C' are given in Table I. The viscosity of the eluent is *ca.* 1 cP, and consequently the inter-diffusion coefficients of the samples in the eluent are higher than in apolar eluents (*e.g.*, heptane or dichloromethane with a viscosity of *ca.* 0.45 cP). The measured efficiencies are in agreement with the calculated values[16,17]. A typical separation at a linear velocity of $u = 4$ mm/sec is shown in Fig. 2.

TABLE I

CONSTANTS IN THE EQUATION $h = A' + C'u$

Conditions as in Fig. 1.

Sample	k'	A' *(μm)*	C' *(msec)*
Nitromethane	0	77	4.3
Naphthalene	0.43	71	6.2
Pyrene	1.28	55	11.7
Chrysene	2.21	59	12.1
3,4-Benzfluoranthrene	3.37	67	12.6

Measurement of short-time averaged flow-rate

Control of constant flow-rate by feedback (equipment Ia and Ib). The variation of the flow-rate for the equipment Ia and Ib will be discussed together. In both types of equipment the pumps were in good condition and operated for more than 8 h per

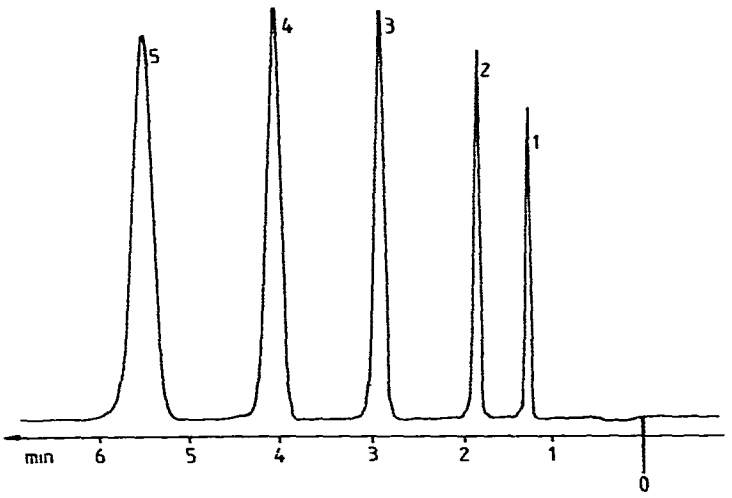

Fig. 2. Separation of polynuclear aromatic hydrocarbons. Conditions as in Fig. 1, except $u = 4$ mm/sec; sample sizes: 1–2 μg per compound. h (μm): (1) 101; (2) 99; (3) 98; (4) 102; (5) 131. Peaks as in Fig. 1.

day for 4 months. It should be pointed out that the variations of the flow-rate, as discussed below, are also typical of the old pumps of equipment Ia. If there are problems with a pump, the fluctuation of the flow-rate increases suddenly by a significant factor (*e.g.*, 5–10 or more). As shown in Table II, for a given flow-rate the peak areas, A, and the relative peak areas, α, were determined a minimum of 10 times. In Table II, A_3 is the peak area in count units as printed by the integrator and α_3 the reduced peak area in percentage units for compound No. 3, *i.e.*, pyrene. From experience, statistically one in 10 (or 20) measurements was "wild", for example because of the formation of air bubbles due to the injection, and the deviations from the average then became extremely high because of this systematic error in the ex-

TABLE II

PEAK AREAS (A) IN ABSOLUTE COUNTS AND REDUCED AREAS (α) IN %

Equipment, Ib; $F = 2$ ml/min.

Run	A_2	A_3	A_4	A_5	α_2	α_3	α_4	α_5
1	51 110	97 940	192 600	275 400	8.28	15.87	31.21	44.63
2	51 150	98 320	192 900	274 200	8.29	15.95	31.29	44.47
3	50 980	98 420	192 900	274 200	8.27	15.96	31.29	44.47
4	51 390	98 780	192 900	274 000	8,33	16.01	31.26	44.40
5	51 270	98 440	193 100	273 500	8.32	15.97	31.33	44.38
6	51 760	98 760	193 500	275 400	8.28	15.96	31.26	44.49
7	51 020	99 960	194 900	278 000	8.29	16.00	31.20	44.51
8	51 550	98 500	194 100	276 400	8.23	15.89	31.30	44.58
9	50 930	98 180	192 700	274 000	8.36	15.93	31.26	44.45
10	51 300	97 960	192 200	273 800	8.29	15.93	31.26	44.52
Average	51 246	98 526	193 180	274 890	8.29	15.94	31.26	44.49
σ (%)	0.6	0.6	0.4	0.5	0.4	0.3	0.1	0.2

periment. These, and only these, results were neglected. In Table II only the results for compounds that are solids at room temperature (*i.e.*, samples 2–5 in Table I) are given in order to avoid errors resulting from fractional evaporation of the sample mixture. As shown in the last line in Table II, the standard deviations in percentage units, σ_A, for the area A for a given compound are greater than those for the relative area, σ_α. As shown in eqns. 10 and 12, the reproducibility of the peak areas, A, is also a function of the reproducibility of the sample size, while the error in α is caused solely by variations of the flow-rate.

Table III gives the average peak areas, A, for given flow-rates and compounds, and their standard deviations, σ_A. In the last two columns the average reduced areas, α, and their standard deviation, σ_α, are tabulated. As shown in eqn. 12, the variation in flow-rate is identical with (or smaller than) the variation in the reduced peak areas, σ_α. From the last column in Table III it follows that, at a constant flow-rate, its variation is more or less independent of the capacity ratio (or retention time) of the compound.

TABLE III

REPRODUCIBILITY AS A FUNCTION OF THE FLOW-RATE WITH EQUIPMENT Ia

F (ml/min)	u (mm/sec)	Compound number	$\bar{A}$	σ_A (%)	$\bar{\alpha}$	σ_α (%)
1	2	2	11 910	2.1	10.61	0.4
		3	22 889	2.2	20.37	0.4
		4	33 345	2.3	29.65	0.6
		5	44 214	2.1	39.32	0.4
2	4	2	604.4	2.3	10.54	0.5
		3	1 171.8	2.3	20.44	0.6
		4	1 689.5	2.5	29.48	0.4
		5	2 265.9	2.8	39.53	0.5
4	8	2	365.7	1.8	10.13	0.4
		3	740.3	1.9	20.52	0.2
		4	1 073.87	1.9	29.78	0.2
		5	1 426.4	1.9	39.55	0.1
6	12	2	251.7	1.7	10.16	1.3
		3	506.9	2.4	20.47	0.5
		4	732.2	2.5	29.58	0.5
		5	984.5	2.6	39.78	0.6

The variation of the short-time averaged flow-rate is independent of the flow-rate and is (with one exception) always less than $\pm 0.6\%$. This seems to be an excellent value, but its consequence is that with such an equipment the reproducibility of a quantitative analysis never can be better than $\pm 0.6\%$. This is also true if internal standards are used.

The value of σ_α at a flow-rate of 6 ml/min for naphthalene (No. 2), with $k' = 0.43$, is high (1.3%), as a consequence of a systematic error. There is a small detector signal for the methanol in which the injected sample is dissolved and, because of the small capacity ratio of naphthalene at high flow-rates, the integrator does not

resolve these two peaks perfectly. This systematic error can be avoided if a more sophisticated integrator is used.

In Table IV, data obtained with equipment Ib are given. The results are similar to those in Table III, except that the variations at $F = 1$ ml/min are definitely higher than at higher flow-rates, where the deviation from the short-time averaged flow-rate is less than $\pm 0.4\%$. This is probably a consequence of the different principles of flow-rate regulation used in the pumps in equipment Ia and Ib.

TABLE IV

REPRODUCIBILITY AS A FUNCTION OF THE FLOW-RATE WITH EQUIPMENT Ib

F (ml/min)	u (mm/sec)	Compound number	$\bar{A}$	σ_A (%)	$\bar{a}$	σ_a (%)
1	2	2	104 777	0.8	8.19	0.9
		3	201 766	0.6	16.12	0.6
		4	396 500	0.8	31.04	0.7
		5	573 688	0.6	44.94	0.4
2	4	2	51 246	0.6	8.29	0.4
		3	98 526	0.6	15.95	0.3
		4	193 180	0.4	31.27	0.1
		5	274 890	0.5	44.91	0.2
4	8	2	25 900	0.5	8.33	0.4
		3	49 600	0.4	15.91	0.3
		4	97 528	0.5	31.27	0.3
		5	138 822	0.3	44.49	0.3
6	12	2	17 205	0.4	8.40	0.1
		3	32 377	0.3	15.81	0.3
		4	64 089	0.4	31.30	0.2
		5	91 124	0.4	44.53	0.4

The variations of the long-time averaged flow-rates were also determined for both types of equipment by measuring the reproducibility of the retention times for all of the compounds at different flow-rates. The reproducibility was, of course, better and never exceeded $\pm 0.2\%$.

Simple method for checking the quality of the gradient system. Both types of equipment (Ia and Ib) also included a programmer for gradient elution. After the variation of F with the single pump had been determined, two pumps were used in each unit, both delivering the same eluent. A linear "programme" was used, the sum of the flow-rates of the two pumps being kept constant. At the beginning of the programme the first pump delivered 20% and the second 80% of the overall flow-rate, and *vice versa* at the end of the programme. This programme was chosen because the supplier of equipment Ib does not recommend starting with a zero flow-rate of one of the pumps. Absolute and relative peak areas were measured, and some of the results are given in Table V. If the corresponding σ_a values in Tables III–V are compared, it can be seen that the variations are very similar. Consequently, the reproducibility of the gradient programming unit is better than the flow variation of the pump itself. It is remarkable in Table V that the σ_a value of compound No. 2 at

TABLE V

REPRODUCIBILITY OF THE PEAK AREAS WITH "GRADIENT PROGRAMME"

Equipment	*F (ml/min)*	*Programme period (min)*	*u (mm/sec)*	*Compound number*	$\bar{A}$	σ_A *(%)*	$\bar{a}$	σ_a *(%)*
Ia	2	8	4	2	5 051.3	0.7	9.98	0.9
				3	10 115.4	1.5	19.97	0.5
				4	18 011.8	1.6	35.60	0.6
				5	17 430.1	0.2	34.44	0.3
Ia	4	4	8	2	2 545.4	0.7	10.22	0.1
				3	5 000.0	0.8	20.10	0.7
				4	8 623.3	1.7	34.64	0.9
				5	8 718.8	0.9	35.02	0.5
Ib	2	8	4	2	53 555	0.5	8.18	0.5
				3	111 744	0.5	17.10	0.2
				4	199 466	0.4	30.52	0.2
				5	288 722	0.3	44.18	0.2
Ib	4	4	8	2	26 918	2.5	8.16	2.0
				3	55 532	1.3	16.85	0.3
				4	100 620	1.6	30.52	0.4
				5	146 540	1.4	44.35	0.3

$F = 4$ ml/min is high with equipment Ib. Similar problems are discussed in Table III with equipment Ia.

Pump with constant inlet pressure. The fluctuation of the short-time averaged flow-rate was maximal with equipment IIa, as shown in Table VI. The pressure-regulating system has many advantages, as discussed before, however, for quantitative analysis it is not recommended.

TABLE VI

REPRODUCIBILITY OF THE PEAK AREAS WITH EQUIPMENT IIa (CONSTANT INLET PRESSURE)

F (ml/min)	*u (mm/sec)*	*Compound number*	$\bar{A}$	σ_A *(%)*	$\bar{a}$	σ_a *(%)*
1	2	2	713.51	2.2	11.23	1.2
		3	1 403.01	2.5	22.09	1.4
		4	1 792.98	5.6	28.06	3.2
		5	2 467.10	2.0	38.84	1.6
3	6	2	225.73	3.7	11.04	0.7
		3	437.13	2.7	21.39	0.4
		4	605.23	5.3	29.71	1.0
		5	773.20	2.7	37.83	1.0
6	12	2	139.40	3.6	10.48	6.2
		3	270.24	4.4	20.39	6.0
		4	404.26	9.4	30.94	5.4
		5	497.45	6.9	37.32	4.4

Pump with pulse-damping unit. The pulses are damped with a Bourdon-type PTFE tube inserted in oil with a maximal volume of 3.6 ml at 500 atm. This tube connects the pump with the inlet of the column. The pressure drop over this unit is negligible. The results obtained with equipment IIb are given in Table VII.

TABLE VII

REPRODUCIBILITY OF THE PEAK AREAS WITH EQUIPMENT IIb (PULSE DAMPING UNIT)

F (ml/min)	u (mm/sec)	Compound number	$\bar{A}$	σ_A (%)	$\bar{a}$	σ_a (%)
1	2	2	5 908.7	1.3	11.06	1.1
		3	11 737.2	1.0	21.98	1.5
		4	15 057.2	7.2	28.17	5.1
		5	20 691.9	1.1	38.76	2.5
3	6	2	2 354.3	4.5	11.36	4.0
		3	4 624.3	3.8	22.32	2.2
		4	5 486.4	5.4	26.47	3.3
		5	8 252.2	3.6	39.82	1.0
6	12	2	1 164.4	1.9	10.98	1.6
		3	2 308.2	1.6	21.77	1.4
		4	2 986.9	3.0	28.16	1.5
		5	4 142.8	1.9	39.07	0.3

As demonstrated in Tables III–VII, the variations of the short-time averaged flow-rate with flow-controlled pumps (equipment Ia and Ib) are less than *ca.* $\pm 0.5\%$, whereas the variations with the other pumps are greater than $\pm 1\%$. Consequently, controlled flow-rate pumps are to be preferred for quantitative separations in HPLC.

Reproducibility of the mass of the sample. It follows from eqns. 10 and 12 that the reproducibility of the sample injection is, to a good approximation (*i.e.*, assuming that all other errors in quantitative work are avoided), equal to the difference between σ_A an σ_a. From the data in Tables III and IV, it follows that the reproducibility of the sample injection averages *ca.* $\pm 1.7\%$ with equipment Ia and $\pm 0.2\%$ with equipment Ib. The averages for all of the manual injections (*i.e.*, equipment Ia, IIa and IIb) are similar, *i.e.*, less than $\pm 2\%$. With longer experience than our operator had had, the "manual" reproducibility can be reduced to $\pm 1\%$. An advantage of manual injection is that the sample size can be varied continuously over a wider range than with the automatic sampling system. In quantitative analysis, the reproducibility of the sample size is of lesser interest than that of the flow-rate if the peak area method is used. Whether manual or automatic sampling systems are to be preferred depends on the particular problems to be solved.

Problems in programmed quantitative analyses

In chromatography, optimal rather than maximal resolutions, R, are required. A better separation than a baseline separation ($R = 1.5$) results only in an increased time of analysis and a decreased signal-to-noise ratio at the peak maxima. In order to decrease the resolution, the temperature, the flow-rate or the composition of the

eluent can be varied as a function of time. The problems that occur in temperature and gradient programming are well known[18]. If the retentions do not need to be reduced too greatly, flow programming is the simplest method[19]. If the equipment includes a gradient programming unit, flow programming is always possible by using only one of the two pumps. Increasing the flow-rate as a linear function of time is the simplest programme:

$$F = F_0 + ft \tag{14}$$

where F_0 and F are the flow-rates when starting the programme and after a time t, respectively, and f is the constant of the linear programme. The long-time averaged reproducibility of the flow programme can be determined by measuring the reproducibility of the retention time, t_R, of a given compound.

The reproducibility of a quantitative analysis with gradient elution can never be better than that with flow programming when the same equipment is used. Quantitative analysis with flow programming requires, however, a short-time and not a long-time averaged "constancy" of the flow-rate, as discussed before.

If the peak area of a given compound in the mixture to be separated is A_i at a constant flow-rate F_i, and A_i^* at $\bar{F}$, and if $\bar{F}_i$ is the flow-rate at the peak maximum, *i.e.*, t_R time units after the flow programme was started, then assuming that the sample size, m, is the same in both analyses and the response factor, r, is of course constant, it follows from eqn. 6, if the flow programme is linear, that

$$A_i F_i = A_i^* \bar{F}_i \tag{15}$$

The reduced area for A_i^* is

$$\alpha_i^* = \frac{A_i^*}{\Sigma A_i^*} \tag{16}$$

If eqns. 15 and 16 are combined, we obtain

$$\bar{F}_i = F_i \cdot \frac{\alpha_i}{\alpha_i^*} \cdot \frac{\Sigma A_i}{\Sigma A_i^*} \tag{17}$$

If only the reproducibility of $\bar{F}_i$ is of interest, F_i, α_i and ΣA_i are constants in eqn. 17. As shown in eqn. 12, the variation of the flow-rate is proportional to α^*. Unfortunately, the reproducibility of the sample size (ΣA_i^*) is also included in eqn. 17. In Table VIII, the variations of $\bar{F}$ are given, and the second column gives the initial and final flow-rates. The final flow-rate was always achieved after the last peak had emerged from the column. In the fourth column the constant f in eqn. 14 (*i.e.*, the acceleration of the flow) is tabulated. The standard deviations of $\bar{F}$ are given in the last four columns for compounds 2–5 for equipment Ia and Ib. From the standard deviations of $\bar{F}$ itself, $\pm 1.7\%$ for equipment Ia and 0.2% for Ib are subtracted, because these are the values for the reproducibility of the sample size, as discussed before. This is, of course, an extremely rough approximation.

TABLE VIII

CALCULATED STANDARD DEVIATIONS (%) FOR THE PROGRAMMED FLOW-RATE, F

Equipment	F *(ml/min)*	$t_{R,5}$ *(min)*	f *(ml/min²)*	*Compound*			
				2	*3*	*4*	*5*
Ia	1–2	7.7	0.125	1.2	2.3	1.9	4.0
Ib	1–2	7.7	0.125	0.5	0.3	0.0	0.3
Ia	2–4	4.1	0.4	1.5	1.8	4.3	1.9
Ib	2–4	4.1	0.4	3.8	3.4	3.1	2.8
Ia	4–6	2.4	0.666	0.0	0.0	0.1	0.0
Ib	4–6	2.4	0.666	3.9	4.5	4.0	3.9

A typical flow-programmed separation is shown in Fig. 3. The programme was chosen so as to achieve optimal separation conditions while keeping all other errors, except that of the short-time variation of the programmed flow-rate, at a minimum. The flow programme was started with the injection of the sample, because this programme had to be tested.

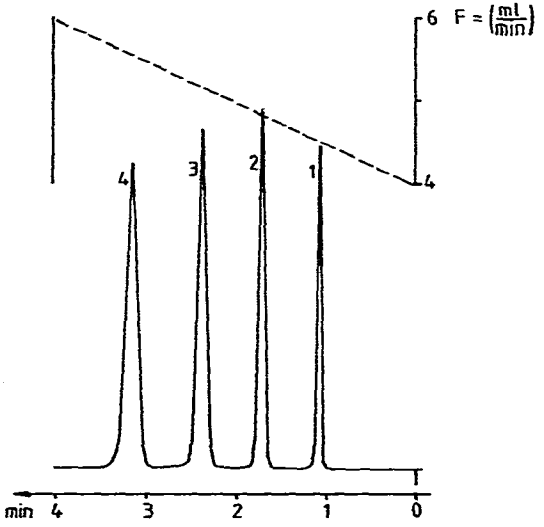

Fig. 3. Flow-programmed separation. Conditions as in Fig. 1, except initial flow-rate = 2 ml/min and final flow-rate = 4 ml/min. Linear flow programme as shown by the dotted line. Peaks: 1 = naphthalene; 2 = pyrene; 3 = chrysene; 4 = 3,4-benzfluoranthene.

As can be seen in Table VIII, the short-time fluctuations (*i.e.*, during the time when the peak emerges from the column) are not only a function of the retention time of the peaks, but also change with the flow-rate and its acceleration, f. Depending on the experimental conditions chosen, either equipment Ia or Ib shows advantages or disadvantages compared with the other.

In Table IX, the average values of the standard deviations of the short and long-time averaged fluctuations of the programmed flow-rate are given. In the fifth

and sixth columns the average values of each line in Table VIII are given. In the last two columns of Table IX the differences between the retention times as calculated for an ideal flow programme and the measured values are given. The last column is also averaged over the capacity ratios of the compounds for a given programme. To determine the averages, a minimum of 10 measurements were made for all flow programmes. Both deviations in the last two columns in Table IX show the same trend and, depending on the programme, either equipment Ia or Ib is to be preferred.

TABLE IX

SHORT- AND LONG-TIME AVERAGED VARIATIONS OF THE PROGRAMMED FLOW-RATE

Equipment	F *(ml/min)*	$t_{R,5}$ *(min)*	f *(ml/min²)*	σ (%) of $\bar{F}$			
				Area		Δt_R	
a	1–2	7.7	0.125	2.4		1.3	
b	1–2	7.7	0.125		0.3		0.2
Ia	2–4	4.1	0.4	2.4		2.4	
Ib	2–4	4.1	0.4		3.3		2.4
Ia	4–6	2.4	0.666	0.0		0.0	
Ib	4–6	2.4	0.666		4.1		2.3

It should be stressed that in gradient elution with a constant overall flow-rate, the flows of two pumps are programmed. The variations in the flow programme seem to compensate each other, as can be seen by comparing Tables V and IX. The variations of the flow-rates of both eluents in gradient elution not only result in deviations of the peak areas but the retentions can also be changed by this effect. Furthermore, the "constant" flow-rate in gradient elution can change, because of the heat of mixing (*i.e.*, viscosity of the mixture) and because of volume contraction or dilation due to the mixing procedure. This effect is great with extremely non-ideal mixtures, for example for the methanol–water system often used in routine work. Extreme caution is essential if quantitative analysis is carried out with the peak area method using the gradient elution technique.

CONCLUSIONS

In HPLC, the concentration of the sample in the eluent at the peak maximum is usually smaller than 50 ppm, and consequently more sensitive detectors are required than in gas chromatography. Provided that the inner diameter of the columns (with the usual lengths) is greater than 3 mm, detectors with a cell volume of 10 μl are acceptable for quantitative analysis. The linearity range of a spectroscopic detector is determined by, among other factors, the validity of the Lambert–Beer law. If an exact quantitative analysis is required, the linear range of a UV detector with a constant wavelength has to be determined for every compound. The optimal approach, of course, is to shift the wavelength of a UV detector to measure at the maximum of the UV absorption of each component of the mixture.

Variations in the flow-rate, F, during the period when the separated peaks are in the detector (*i.e.*, short-time averaged flow-rate), and only during this period, give erroneous results in quantitative analysis, even if an internal standard is used. A simple experimental method is proposed for measuring the short-time averaged variations of the flow-rate and the reproducibility of the sample injection. The reproducibility of the short-time averaged flow-rates for two commercially available types of equipment (Ia and Ib) are less than $\pm 0.6\%$ if there is a feedback for the flow control. The long-time averaged flow-rates are better than $\pm 0.2\%$. Equipment with a constant inlet pressure (IIa) is not recommended for quantitative work.

The reproducibility of the sample size injected is better than $\pm 0.2\%$ if an automatic injection system is used.

The reproducibilities as discussed above are optimal values. Not only were the stationary phase and the eluent of high quality, but also the sample was mixed so as to achieve baseline-resolved peaks with roughly equal heights. In routine quantitative analysis, such parameters are extremely improbable. Consequently, quantitative analysis in HPLC with $\pm 1\%$ reproducibility seems to be excellent, if the peak area method is used. It seems to be questionable whether the peak height method is more reproducible.

A simple as well as a more sophisticated experimental method is proposed for checking the quality of the gradient-generating systems, by which the reproducibility of the flow programme of the pump(s) is determined. The reproducibility of quantitative analyses with the gradient elution method never can be better than that of the flow programme.

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SYMBOLS

a = εcd = absorbance (optical density)
b = height of the triangular-shaped peak (in concentration units)
c = concentration of the sample in the eluent
d = thickness of the cell
d_p = average particle size of the support
f = constant in eqn. 14
h = height equivalent to a theoretical plate

$k' = \frac{t_R - t_0}{t_0}$ = capacity ratio

m_i = mass of the ith sample component
r = response factor of the detector
t = time
t_R = retention time
u = linear velocity of the eluent

w = base width of the triangular-shaped peak (in times units)
A = peak area
A' = constant in eqn. 13
A^* = peak area with flow programme
C' = constant in eqn. 13
F = mass flow-rate of the eluent
F_0 = flow-rate at the start of the flow programme
$\bar{F}$ = mass flow-rate of the eluent at t_R in a linear flow programme
F_1 = mass flow-rate of the sample
I = intensity of light for the sample and eluent
I_0 = intensity of light for the eluent
K = specific permeability
L = column length

$$R = \frac{\Delta t_R}{w} = \text{resolution}$$

S = detector signal
V_s = volume of sample injected
$\alpha_i = A_i/\Sigma A_i$ = reduced peak area
α_i^* = reduced peak area with flow programme
ε = extinction coefficient of the sample in eqn. 4
ω = standard deviation of a gaussian peak in volume units of the eluent.

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